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Moderate hyperhomocysteinemia provokes dysfunction of cardiovascular autonomic system and liver oxidative stress in rats

R.H. Mendes [a,f], C. Mostarda [b,c], G.O. Candido [c], I.C. Moraes-Silva [c,g], V. D'Almeida [e], A. Belló-Klein [a], M.C. Irigoyen [a,c], K. Rigatto [a,d,*]

[a] Laboratório de Fisiologia Cardiovascular, Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Brazil
[b] Hospital Materno Infantil, Universidade Federal do Maranhão, Brazil
[c] Unidade de Hipertensão, Instituto do Coração, Universidade de São Paulo, Brazil
[d] Universidade Federal de Ciências da Saúde de Porto Alegre, Brazil
[e] Departamento de Biociências, UNIFESP, SP, Brazil
[f] Instituto de Nutrição Josué de Castro, Universidade Federal do Rio de Janeiro, Brazil
[g] Programa de Pós-Graduação em Ciências da Saúde, Universidade Santo Amaro, Brazil

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ABSTRACT

Hyperhomocysteinemia (HHcy) is associated with cardiovascular disease, atherosclerosis and reactive oxygen species generation. Thus, our aim was to investigate whether there was an association between HHcy, blood pressure, autonomic control and liver oxidative stress. Male Wistar rats were divided into 2 groups and treated for 8 weeks: one group (control, CO) received tap water, while the other group (methionine, ME) was given a 100 mg/kg of methionine in water by gavage. Two catheters were implanted into the femoral artery and vein to record arterial pressure (AP) and heart rate (HR) and drug administration. Signals were recorded by a data acquisition system. Baroreflex sensitivity was evaluated by HR responses to AP changes induced by vasoactive drugs. HR variability and AP variability were performed by spectral analysis in time and frequency domains to evaluate the contribution of the sympathetic and parasympathetic modulation. Lipid peroxidation and antioxidant enzyme activities were evaluated by measuring superoxide dismutase, catalase and glutathione peroxidase in liver homogenates. The ME group presented a significant increase in systolic arterial pressure (118 ± 9 vs 135 ± 6 mm Hg), diastolic arterial pressure (81 ± 6 vs. 92 ± 4) and mean arterial pressure (95 ± 7 vs. 106 ± 6). In addition, pulse interval variability presented a significant decrease (41%), while the low frequency component of AP was significantly increased (delta $P = 6.24$ mm Hg^2) in the ME group. We also found a positive association between lipid peroxidation and cardiac sympathetic modulation, sympathetic and vagal modulation ratio and systolic pressure variability. Collectively, these findings showed that HHcy induced dysfunction of cardiovascular autonomic system and liver oxidative stress.

1. Introduction

Homocysteine (Hcy) is a sulfur amino acid that is derived from dietary methionine, which is synthesized during metabolic conversion of methionine to cysteine in the liver (Medina et al., 2001; Selhub, 2002). Plasma Hcy levels may be altered by genetic and nutritional factors (Refsum et al., 1998).

Hyperhomocysteinemia (HHcy) is usually defined by level of Hcy: (>12 and <30 μmol/L) moderate, (>30 and <100 μmol/L) intermediate and (100 μmol/L) severe (De Bree et al., 2002; Van Guldener et al., 2003). HHcy occurs in about 5–7% of the world population (Seshadri et al., 2002; Suematsu et al., 2007).

Moderate HHcy (12–30 μmol/L) is prevalent and an independent risk factor for cardiovascular disease and atherosclerosis. The precise underlying mechanism that would account for the relationship between Hcy and atherosclerosis remains unclear. However, studies using genetic- and diet-induced animal models of HHcy show a direct causal relationship between HHcy, endothelial dysfunction and accelerated atherosclerosis (Lawrence de Koning et al., 2003).

Physiopathological features of HHcy are similar to some physiopathological features found in sympathetic overactivity in the cardiovascular system. Both are characterized by increased platelet aggregation, proliferation of vascular smooth muscle, accelerated atherosclerosis, left ventricular hypertrophy and arterial hypertension (Bortolotto et al., 1999). It is well documented in the literature that HHcy provokes an activation of the sympathetic system, and this fact contributes to vascular and end-

* Corresponding author at: Universidade Federal de Ciências da Saúde de Porto Alegre, R. Sarmento Leite, 245, Cidade Baixa, Zip Code 90050-170, Porto Alegre, RS, Brazil. Tel.: +55 5133308753.
E-mail address: krigatto@gmail.com (K. Rigatto).

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organ damage (Grassi et al., 1995; Nygard et al., 1997; Bortolotto et al., 1999; Muntzel et al., 2006).

HHcy induced by methionine supplementation is also associated with the liver. The increase of methionine might lead to liver oxidative/nitrosative stress increment and Hcy itself has the ability to generate potent reactive oxygen species (ROS) when oxidized by highly reactive sulfhydryl group (Yamada et al., 2012). Another mechanism is the HHcy-induced endothelial injury throughout Hcy oxidation that generates homocysteine, leading to the generation of superoxide and hydrogen peroxide (Steed and Tyagi, 2011). In fact, in vitro studies or experimental models have associated HHcy with oxidative stress (Lubos et al., 2007; Mendes et al., 2010).

Establishing a solid association between HHcy, liver oxidative stress and blood pressure variability is critical for development of new therapeutic strategies for hypertension. We would like to stress that no previous study has so far evaluated the effects of methionine supplementation on cardiovascular autonomic system and baroreflex associated with liver oxidative stress.

Therefore, the aim of this study was to investigate the effects of HHcy on blood pressure and sympathetic and parasympathetic functions and the impact about some oxidative stress parameters in the liver.

2. Material and methods

2.1. Animals and experimental groups

Twelve male Wistar rats (250–300 g) from the Federal University of São Paulo, Brazil were housed in plastic cages (3 animals each), with access to water and regular food ad libitum, and maintained under controlled temperature (21 °C) and 12 h light/dark cycle. They were divided in 2 groups and treated by a daily gavage: 1) control (CO) receiving water only and 2) methionine (ME) receiving methionine (100 mg/kg) diluted in water. After 8 weeks, animals were killed by decapitation. All animal procedures were carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication no. 85-23, revised 1985).

2.2. Determination of plasma Hcy concentration

Total Hcy concentration in plasma was measured by high-performance liquid chromatography with fluorescence detection (de Oliveira et al., 2002).

2.3. Hemodynamic measurements

One day after the end of treatment, 2 catheters filled with 0.06 mL saline were implanted into the femoral artery and vein (PE-10) of anesthetized rats (50 mg/kg ketamine and 10 mg/kg xylazine) for direct measurements of AP and drug administration, respectively. Rats receiving food and water ad libitum were studied 1 day after catheter placement; the rats were conscious and allowed to move freely in the cage during the experiments. The arterial cannula was connected to a strain gauge transducer 6 (P23Db, Gould-Statham), and blood pressure signals were recorded over a 20-minute period by a microcomputer equipped with an analog-to-digital converter board (using a Dataq Instruments DI-720, 16-bit measurement resolution, 250 Hz sampling rate). WINDAQ/PRO waveform recording software was used for the acquisition at 2000 Hz per channel.

The recorded data were analyzed on a beat-to-beat basis to quantify changes in mean (MAP), systolic (SAP) and diastolic arterial pressure (DAP) and heart rate (HR). Increasing doses of phenylephrine (0.25 to 32 μg/kg) and sodium nitroprusside (0.05 to 1.6 μg/kg) were given as sequential bolus injections (0.1 mL) to produce blood pressure responses ranging from 5 to 40 mm Hg. A 3 to 5-minute interval between doses was necessary for blood pressure to return to its baseline. Peak increases or decreases in MAP after phenylephrine or sodium nitroprusside injection and the corresponding peak reflex changes in HR were recorded for each dose of the drug. Baroreflex sensitivity (BRS) was evaluated by a mean index through a relationship between changes in HR and in MAP, allowing separate analysis of bradycardia and tachycardia reflexes. The mean index was expressed as beats per minute per millimeter of mercury (Farah et al., 1999).

2.4. Blood pressure and pulse interval variability

Time-domain analysis consisted of calculating the mean pulse interval (PI) and systolic arterial pressure (SAP), as well as their variability as the standard deviation from their respective time series. In the frequency-domain analysis, Fast Fourier Transforming (FFT) method was used to evaluate SAP, PI and RR interval variability (SBPV and PIV, respectively). The spectral bands for rats (very low frequency (VLF): 0.0–0.2 Hz; low frequency (LF): 0.2–0.75 Hz; high frequency (HF): 0.8–2.8 Hz) were defined according to the literature (Grassi et al., 1995; Hiratzka, 1996).

Spectral power for LF and HF bands was calculated by means of power spectrum density integration within each frequency bandwidth. The power density of each spectral component was calculated both in absolute values and in normalized units. Power in LF and in HF for pulse intervals was normalized by calculating the variance minus the power in very low frequency (VLF) and was expressed in normalized units. The sympathetic and vagal modulation ratio balance was defined by the LFnu/HFnu ratio.

The LF components of the PIV and LF components of the SBPV were considered markers of efferent sympathetic cardiac and vascular modulation, respectively, whereas the HF component of the PIV reflected respiratory-driven vagal modulation to the sinoatrial node. For frequency-domain analysis, the entire ten-minute time series of blood pressure and pulse, or RR intervals, were evaluated under basal conditions using non-parametric methods (FFT), described in detail above. Beat-to-beat values of SBP and PI intervals were used to estimate the cardiac BRS by spectral analysis, using the alpha index for the low-frequency band (0.20–0.75 Hz). The alpha index analysis evaluates short-term changes in the systolic arterial pressure and in the RR interval.

The coherence between the PI and the SBP signal variability was assessed by means of a cross-spectral analysis. The alpha index in the LF band was calculated only when the magnitude of the squared coherence between the PI and SBP signals exceeded 0.5 (range, 0–1). After coherence calculation, the alpha index was obtained from the square root of the ratio between PI and SBP variability in the two major LF bands (Soares et al., 2004; Moraes-Silva et al., 2013).

2.5. Tissue preparation

Livers were rapidly excised, weighed and homogenized (1.54 mol/L potassium chloride, phenylmethyl sulfonyl fluoride 20 mmol/L) in ULTRA-TURRAX (Fisher Scientific, Canada). The suspension was centrifuged at 6000 *g* for 10 min at 0 °C to 4 °C to remove the nuclei and cell debris. The supernatant was stored at −80 °C for biochemical analysis.

2.6. Determination of oxidative stress parameters

Catalase (CAT) activity was determined by following the decrease in hydrogen peroxide (H_2O_2) absorbance at 240 nm. It was expressed as nanomoles of H_2O_2 reduced per minute per milligram of protein (Aebi, 1984). Superoxide dismutase (SOD) activity, expressed as units per milligram of protein, was based on the inhibition of superoxide radical reaction with pyrogallol (Marklund, 1985). GSHPx activity was determined by monitoring NADPH oxidation spectrophotometrically at 340 nm, and the results are reported as nmol/min/mg protein (Flohe and Gunzler, 1984).

Lipid peroxidation was determined by tert-butyl hydroperoxide initiated chemiluminescence (CL) by scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB-Produkter AB, Sweden) (Gonzalez Flecha et al., 1991).

2.7. Protein determination

Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard. The results were expressed in mg of protein/mL.

2.8. Statistical analysis

The data are expressed as mean ± SD. Student's t test was used to compare groups and differences were considered significant when $P<0.05$. Pearson correlation was used to study the association between variables. Statistical analyses were performed using GraphPad InStat 3.02 for Windows (San Diego, California, USA) and Sigma Plot 12.0.

3. Results

3.1. Plasma Hcy concentration

As expected, Hcy concentration in plasma (mol/L) in the ME treated rats (16.81 ± 2.39) was significantly higher than CO (11.95 ± 3.16) ($P>0.05$, n = 4/group).

3.2. Hemodynamic and autonomic parameters

Tables 1, 2 and Fig. 1 show the cardiovascular and spectral measurements. There was a significant increase in SAP, DAP and MAP in animals that received methionine supplementation (ME group). The treatment induced an important reduction in both tachycardic response (TR) and bradycardic response (BR).

PIV presented a significant decrease in animals that received supplementation (ME group). The LF of PIV and systolic arterial pressure variability was significantly increased in the ME group. HF% presented a significant decrease in the ME group while the LF/HF was significantly increased in the ME group. In contrast, the BRS estimated by alpha index was significantly decreased in the ME group. On the other hand, regarding to SAPV, we found a significant increase in LF (3.1 ± 0.5 vs 9.34 ± 6) and a decrease in BRS (1.29 ± 0.5 vs 0.75 ± 0.2) in the ME group as estimated by alpha index (Fig. 1).

3.3. Liver oxidative stress parameters

Changes in lipid peroxidation, GSHPx, CAT and SOD in liver samples are reported in Table 3. There was a significant increase (49 and 66%, respectively) in lipid peroxidation measured by CL and GSHPx activities in animals that received methionine supplementation (ME group). There were no changes in CAT and SOD activities considering treatment.

Table 1
Cardiovascular measurements in experimental groups: hemodynamic variables.

Parameters	CO (n = 6)	ME (n = 6)	P
Hemodynamics			
SAP (mm Hg)	118 ± 9	135 ± 6	0.003
DAP (mm Hg)	81 ± 6	92 ± 4	0.004
MAP (mm Hg)	95 ± 7	106 ± 6	0.01
HR (bpm)	323 ± 17	343 ± 20	0.09
TR (bpm/mm Hg)	2.5 ± 0.5	1.2 ± 0.4	0.0006
BR (bpm/mm Hg)	−2.0 ± 0.5	−1.3 ± 0.2	0.009

Data are reported as means ± SD. Results of systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), heart rate (HR), tachycardic response (TR), bradycardic response (BR).

Table 2
Spectral parameters of pulse interval after methionine treatment.

PI	CO (n = 6)	ME (n = 6)	P
PIV (ms^2)	113 ± 31	66.54 ± 34	0.03
LFa (ms^2)	2.93 ± 1.41	8.30 ± 6.01	0.08
HFa (ms^2)	13 ± 8.0	9.61 ± 3.0	0.35
LFnu (%)	19 ± 4	52 ± 22	0.005
HFnu (%)	80 ± 4	48 ± 22	0.005
LF/HF ratio (ms^2)	0.23 ± 0.05	1.28 ± 1	0.03

Data are reported as mean ± SD. Results of pulse interval variability (PIV), and frequency domains (LF: low frequency, HF: high frequency, LFnu: low frequency normalized, HFnu: high frequency normalized).

3.4. Correlation analysis

Correlation analyses were carried out associating autonomic and cardiac oxidative stress parameters. Regarding the arterial pressure, there was a positive correlation between CL and cardiac sympathetic modulation (LF %), ($r=0.63$ $P\leq0.03$), and also between CL and sympathetic and vagal modulation balance ($r=0.79$ $P\leq0.01$). In addition, CL also showed a positive and significant association with sympathetic modulation of blood pressure (LF of SAP) ($r = 0.76$ $P \leq 0.01$) and blood pressure total variance (SAPV) ($r=0.78$ $P\leq0.004$).

4. Discussion

The main finding of the present study was that the ME supplementation promoted a moderate state of HHcy, which was accompanied by an increase in SAP, DAP and MAP. This increase was also associated with an increase in sympathetic modulation and a decrease in parasympathetic modulation. In addition, there was a positive association between CL and cardiac sympathetic modulation, sympathetic and vagal modulation ratio (LF/HF) and systolic pressure variability (SAPV). Collectively, these results reinforce the hypotheses that the HHcy increased blood pressure and liver oxidative stress.

As previously described by our group, the same protocol of ME supplementation was able to significantly increase the Hcy blood concentration, promoting a moderate HHcy in rats (Mendes et al., 2010). Moreover, our results are in agreement with an experimental study which adopted the strategy of folate deficiency for inducing HHcy. This study also showed a significant increase in SAP, measured by telemetry, associated with increased heart, kidney and liver oxidative stress (Pravenec et al., 2013).

A possible mechanism, which may account for the association between HHcy and increased arterial pressure, is the impairment of vascular endothelial function (He et al., 2010). It is well established that HHcy provokes an increase in vascular thickness and elastin fragmentation and a decrease in vessel wall elastic compliance (Steed and Tyagi, 2011). Combined, these findings may have contributed to the increase in SAP, DAP and MAP in the ME group found in our study that it is in accordance to the autonomic imbalance. In fact, in a previous study with normotensive subjects, we have demonstrated that a 10 mm Hg increase in the mean arterial pressure also increased the LF component participation and the LF/HF ratio (Vargas et al., 2013).

In the present study HHcy also resulted in impaired BRS, an autonomic reflex to buffer beat-to-beat fluctuations in arterial blood pressure (Thomas, 2011), followed by impaired autonomic balance, probably due to an increase in sympathetic modulation. On the other hand, our results did not corroborate an experimental study that found an increase in BRS after 15 days of DL-Hcy thiolactone treatment (Resstel et al., 2008). The difference in the results may be probably due to the time of treatment. In our study, rats underwent a longer exposure to methionine, which may have resulted in impaired vascular function and autonomic modulation, and consequently BRS.

The reduction in spontaneous BRS, seen by alpha index, accompanied by an increase of LF component and heart rate variability and a

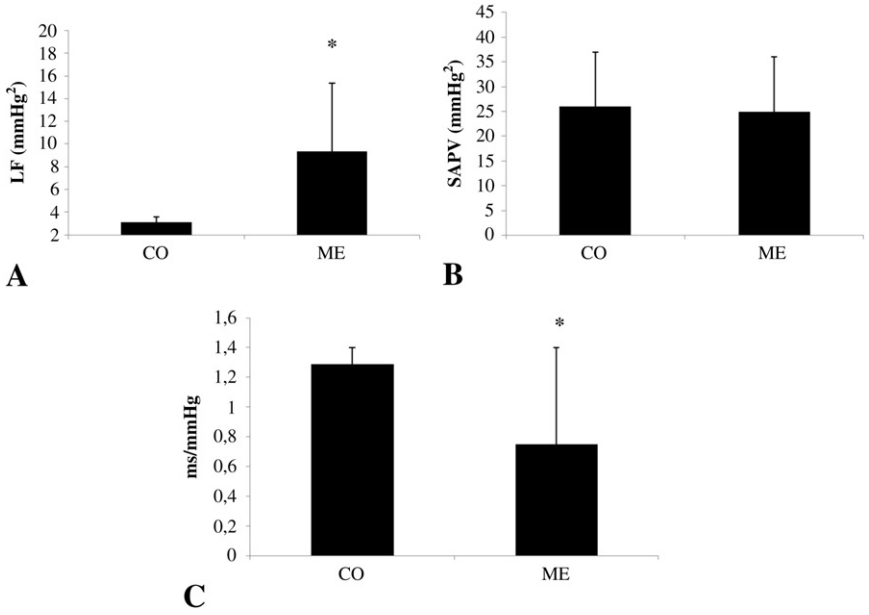

Fig. 1. A: Low frequency band of systolic arterial pressure. B: Systolic arterial pressure variability. C: Spontaneous baroreflex sensitivity evaluated by alpha index. Data are reported as mean ± SD.

decrease in PIV, was observed in the spectral analysis results. Collectively, these findings may confirm that HHcy is associated with increased cardiac and AP sympathetic participation (Ebesunun et al., 2008; Resstel et al., 2008; Zivkovic et al., 2012) and a decrease in pulse interval variability (Hiratzka, 1996).

In fact, it has been demonstrated that the decrease in LF component may also be associated to BRS impairment (Robbe et al., 1987; Farah Vde et al., 2007). This is confirmed by another study of our group, which found an association between the abolishment of BRS and the reduction in LF component of HR variability, resulting in a worse cardiac remodeling (Mostarda et al., 2010). These results lend support to the hypothesis that the efficient functioning of the autonomic nervous system is dependent on a balance between the sympathetic and parasympathetic nervous system. Either an increase or a decrease in any of the autonomic components may cause an impairment of the BRS due to a reduction in the heart capacity to buffer arterial pressure alterations.

Consequences of the autonomic dysfunction associated with the HHcy may include pro-atherogenic effects. Evidence has shown that Hcy is not only related to atherogenic effects, but also to an increased in coagulation (Gerdes et al., 2004). In addition, the atherogenicity of Hcy might be associated with several mechanisms that include the LDL oxidation and the in vitro and in vivo HDL decrease (Xiao et al., 2011).

Moreover, studies have found an association between autonomic modulation and oxidative stress, which may, at least in part, account for the impairment of ANS modulation in the ME group found in our study. An experimental study has shown that NADPH might activate sympathetic nervous system and thus resulting in hypertension (Hirooka et al., 2011). As already demonstrated by other authors HHcy induces endothelial dysfunction through the generation of oxygen free radical and decrease in NO bioavailability (Jin et al., 2007).

Regarding to this association, our results demonstrated significant correlations between CL and LF component of IP, sympathetic and vagal balance and systolic arterial pressure variability, which partially explain the impairment in ANS modulation to the heart and the consequent decrease in BRS.

The liver is the central organ of methionine metabolism, and of the turnover of total body GSH (Lauterburg et al., 1984). Our results showed an increase in liver GSHPx activity measured by spectrophotometry. Glutathione peroxidase-1, a selenocysteine-containing antioxidant enzyme, might be a key target of Hcy's deleterious action (Lubos et al., 2007). One plausible explanation for the increase of GSHPx is an increase of thiol ingestion due to methionine, and a possible overexpression of GPx-1 to compensate the effects of methionine supplementation induced HHcy moderate. Furthermore, the liver oxidative stress results are in agreement with Stefanello et al. (2009) and Fujita et al. (2012), which also found a worsening in the oxidative stress markers after ME treatment and Woo et al. (2006) who have demonstrated a significant increase of malondialdehyde levels in the liver after high-ME diet for 4 weeks. This is probably explained by the increase of lipid peroxidation that is associated with the decrease of antioxidant enzymes and thiol content in the HHcy (Ventura et al., 2000; Racek et al., 2005; Devi et al., 2006).

CAT and SOD activities were not different between control and ME groups. Another study using folate depletion as experimental model of HHcy found no difference between CAT activity and decrease in Cu–Zn SOD during 4 weeks of experimental protocol (Huang et al., 2001). The results of CAT and SOD may be associated with adaptation of antioxidant enzymatic system.

Therefore, these findings provide evidence that moderate HHcy induced by ME impairs the cardiovascular autonomic control which results in increased arterial pressure and it is associated with liver oxidative stress. Collectively, our results reinforce the importance of adopting healthy eating habits, specially increasing the antioxidant food consumption, in order to have a longer and healthy life.

Table 3
Liver oxidative damage and antioxidant enzyme activities after ME treatment.

Parameters	CO (n = 6)	ME (n = 6)
CL (cps/mg prot 10^3)	10.5 ± 2.1	15.6 ± 3.3*
CAT (pmol/mg prot)	162.7 ± 40.7	148.3 ± 54.3
SOD (U/mg prot)	7.8 ± 0.6	7.4 ± 0.3
GSHPx (nmol/min/mg prot)	30.3 ± 11.6	50.2 ± 12.8*

Data are reported as means ± SD. *P < 0.05 vs C. Results of chemiluminescence (CL), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSHPx).

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